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13. ABSTRACT (Maximum 200 Words)

LMO4 is a transcription factor which belongs to the LIM-only (LMO) gene family of oncogenes. We have now shown that expression of LMO4 is associated with undifferentiated cellular stage of breast epithelial cells, such as that found during lobuloalveolar development in pregnancy and in breast cancer. Yet, LMO4 is not induced by estrogen, suggesting that it may participate in an estrogen-independent pathway. Consistent with this idea, we found that LMO4 expression is induced by the Neu/Her2 oncogene in breast cancer cells, suggesting that LMO4 plays a role in this important oncogenic pathway. We created a transgenic mouse model to demonstrate that interfering with LMO4 results in significant inhibition of lobuloalveolar development, indicating that LMO4 plays roles in proliferation and/or invasion of breast epithelial cells. Because these cellular features are associated with breast carcinogenesis, and because LMO4 is overexpressed in a subset of breast cancers, our studies implicate LMO4 as a possible oncogene in breast cancer. On a molecular level, we found that LMO4 functions by interacting with Clim transcriptional co-factors and is likely recruited to DNA in breast cancer cells by the DNA-binding protein GATA3. Expression profiling studies show that LMO4 and Clim regulate several genes involved in breast cancer carcinogenesis. In summary, our studies identify LMO4 as an important regulator of cell growth in normal mammary epithelial cells and in breast cancer cells.

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INTRODUCTION

Understanding the mechanisms involved in regulating proliferation and differentiation of breast epithelial cells is important for further understanding the causes, diagnosis and treatment of breast cancer. We identified a new protein called LMO4, a member of a family of proteins that participate in gene regulation. Proteins belonging to this group have been shown to cause leukemia. We were therefore intrigued to find that LMO4 is highly abundant in breast epithelial cells when these cells are proliferating. Our hypothesis is that LMO4 is involved in regulation of proliferation and/or invasion of epithelial cells in normal breast and in breast cancer. To test this hypothesis, we have pursed the following **Specific Aims**:

#1. Define the expression pattern of LMO4 during normal mouse breast development and in human breast cancer.

#2. Define the role of LMO4 in normal breast development and in breast cancer, using a mouse transgenic approach.

#3. Identify and characterize protein partners for LMO4 in human breast tissue.

BODY

Objective #1. **Define the expression pattern of LMO-4 during normal mouse breast development and in human breast cancer.**

a. Raise and purify LMO-4 antisera.

This task has been accomplished. In summary, we generated LMO4 antisera that are useful for Western blots but were not suitable for immunohistochemistry. After extensive characterization and effort to use these antibodies for developmental studies, we switched to using *in situ* hybridization with LMO4 cRNA probes and RNAse protection assays, as described in alternative approaches in our original proposal.

b. Obtain mouse embryos and pregnant mice at different developmental stages.

We completed collecting fixed embedded mammary glands from mice at different developmental stages for use in immunohistochemistry and *in situ* hybridization studies. We also completed isolation of RNA from mammary glands at different developmental stages for studying LMO4 mRNA levels with RNAse protection assays.

c. Study LMO-4 expression during normal mammary gland development.

This task has been completed and results from RNAse protection assays and *in situ* hybridization studies were featured in our *Oncogene* paper (Fig. 1A and 1B in (1)), which is provided in the Appendix. The results show that LMO4 and Clim2 transcript levels are coordinately and greatly upregulated during mid-pregnancy, a stage in mammary gland development when epithelial cells are undergoing proliferation and invasion into the fat pad. Another expression peak is observed during lactation suggesting possible additional roles during this stage.

Our *in situ* hybridization studies also indicate that LMO4 levels in mammary glands are highest during midpregnancy and become undetectable after weaning, and that LMO4 is mainly expressed within the lobuloalveolar epithelial cells of the mammary gland. Together, these experiments correlate the expression of LMO4 and Clim2 with a stage in development when

breast epithelial cells are relatively undifferentiated and undergoing proliferation and invasion. This expression pattern suggests the possibility that the LMO4/Clim2 complex plays roles in maintaining proliferation, promoting invasion and/or suppressing differentiation – cellular features that characterize breast cancer cells.

d. Obtain human breast and breast tumor samples.

We obtained a panel of cDNAs from breast tumor sections, which are spotted on a nylon membrane suitable for hybridization. These have now been used to study expression of LMO4 in human breast cancer cases.

e. Analyze expression of LMO-4 in normal human breast and breast cancer.

We found that the expression of LMO4 and Clim2 varies markedly between three human breast cancer cell lines (Fig 1C in (1)). LMO4 is greatly overexpressed in one of the breast cancer cell lines, the MDA-MB-231 line, but low in MCF-7 cells. Transcript levels are not regulated by 17-β estradiol in MCF-7 cells. We now have tested the effect of heregulin, which is an ErbB2/Her2/Neu activator, on LMO4 expression in MCF-7 cells (Fig. 1F and G in (1)). Interestingly, heregulin stimulates expression of LMO4 and this effect is partially blocked with an antibody to ErbB2/Her2/Neu, indicating that the effect is mediated via this receptor. These findings are exciting because they link LMO4 to an important oncogene in breast cancer; the ErbB2/Her2/Neu oncogene is overexpressed in 25% of breast cancer cases and predicts a poor prognosis.

Studies from other laboratories (2) have indicated that LMO4 is overexpressed in 50% of cases of breast cancer. Our own studies, using cDNA synthesized from human breast cancers indicates that the situation is more complex (Fig. 1). First, LMO4 levels vary widely in normal breast samples, and second, while LMO4 levels are high in breast cancers, the frequency of overexpression compared to normal breast is lower than 50% and probably more in the neighborhood of 20%.

f. Create and analyze stable MCF-7 breast cancer cell lines in which expression of LMO4 and Clim2 can be induced. (New task)

This task was added to the original proposal based on a request dated November 13, 2002, which was granted by the Army Breast Program. The rationale for this task is to test directly whether LMO4 overexpression contributes to the cancer phenotype of breast cancer cells. The approach for testing the role of LMO4 in breast cancer is to introduce LMO4 into breast cancer cells and ask what happens to gene expression and the biological behavior of breast cancer cells overexpressing LMO4 and its partner Clim2.

We have created inducible MCF-7 cells expressing LMO4, Clim2, or a dominant-negative form of Clim (DN-Clim). Initially, as reported in previous year's Progress Report, we created these cells, using the Tet-on system. However, during long-term culturing, these cell lines did no have stable expression. We then switched to the Tet-off system and successfully created several MCF-7 cell line clones that express LMO4 (Fig. 2, upper panel), Clim2 (not shown) and DN-Clim (Fig. 3, upper panel) in an inducible manner.

We have also made significant progress towards the second part of this task, which was to analyze global changes in gene expression in response to LMO4 and Clim2 expression. Microarray expression analyses have been successful in revealing functionally important signaling pathways in breast cancer (3) and may allow detection of alterations in cluster of genes

carrying out related functions (4, 5). This approach was used to discover important functional pathways regulated by other factors, including BRCA1 (6, 7). We have profiled expression in MCF-7 cells expressing LMO4 in a conditional manner, using the cell lines described in described above (Fig. 2). For these experiments, we selected 3 independent cell clones and profiled expression under basal conditions (control conditions, in the presence of doxycycline) and under induced conditions (LMO4 expression, 7 days after doxycycline withdrawal). To decrease variability, RNA samples from two independent experiments were pooled for each of the three cell clones. We hybridized to U133A and B Affymetrix chips, which contain 44,692 probe sets, and analyzed the data with the Cyber-T program, which was developed at UCI (8). This statistical data package, which is especially suitable for pairwise comparisons, uses a Bayesian statistical framework to determine the local confidence (p-values) based on the *t*-test distribution of individual gene measurements. Thus, for each experimental condition, we can obtain: a mean expression level, a fold-change between control and experimental condition, and a *t*-value to establish the confidence level of the observed difference in expression of a particular gene between control and experimental conditions.

Overview of the data processing is provided in Fig. 2 (lower panel). Using probability criteria of $p \leq 0.05$, 888 probe sets (805 genes) were altered after LMO4 induction. Of these 805 genes, 431 are upregulated and 374 are downregulated. Interestingly, this experiment suggests that not only can LMO4 stimulate gene expression, but also repress a group of genes. Table 1 shows a list of the top genes (listed in order of increasing p value) showing differential expression after induction of LMO4 in MCF-7 cells. Many of the target genes, which we have independently validated with quantitative PCR, are involved in oncogenesis. In an effort to further understand the role of LMO4, we subjected the significantly altered genes to pathway analyses (Ingenuity Systems). The results show (Table 2) that the pathways significantly affected by LMO4 are those involved in cellular proliferation and apoptosis. Therefore, the microarray data from breast cancer cells is consistent with our mouse developmental data: LMO4 plays roles in mammary epithelial cell survival, most likely by affecting cell proliferation. In summary, in human breast cancer cells, LMO4 alters the expression of several genes involved in oncogenesis.

Because LMO4 and Clim are thought to act as a complex, we tested the transcriptional effect of DN-Clim, using the inducible cell line (Fig. 3, top panel). Microarray analysis was performed as described above for the LMO4 cell lines. We found that 579 probe sets (524 genes) were altered by DN-Clim; 337 and 187 genes were upregulated and downregulated, respectively (Fig. 3, lower panel). As predicted, there is significant overlap in the genes regulated by LMO4 and DN-Clim (Fig. 4). However, unexpectedly, the genes that were altered both by LMO4 and DN-Clim expression were all regulated in the same direction, but not in opposite direction as the current models suggest.

Among the target genes upregulated by both LMO4 and DN-Clim were BMP-7 and IGFBP5, which has been implicated in breast cancer. We therefore cloned the promoters of these genes to study the mechanism of transcriptional regulation by LMO4. In transient transfection assays, both LMO4 and DN-Clim upregulated the BMP-7 and IGFBP5 promoters, suggesting that the regulation by LMO4/Clim is direct on these genes (Figs. 5 and 6). Consistent with this finding, LMO4 was found to associate with the BMP-7 and IGFBP5 promoters in chromatin immunprecipitation assays (Fig. 7). These findings have allowed us to create new models for how LMO4 may act on a transcriptional level in breast cancer cells (Fig. 8). Based on our work, we propose that LMO4 disrupts Clim-containing complexes; when these complexes contain

repressors, the disruption results in activation of gene expression and when these complexes contain co-activators, the disruption results in repression of gene expression.

Ojective #2. **Define the role of LMO-4 in normal breast development and in breast cancer, using a transgenic approach.**

Two previously characterized members of the LMO-family, LMO1 and LMO2, have been found to be oncogenic. In humans these genes are overexpressed in lymphocytes due to fusion with the T-cell receptor in chromosomal translocations associated with acute lymphoblastic leukemia. These observations suggest that the LMO class of proteins plays roles in regulation of both proliferation and differentiation critical for organ development and that abnormality in LMO activity may lead to oncogenesis. Our hypothesis is that LMO4 plays a role in normal breast development and that subversion of LMO4 function or activity may contribute to formation of breast tumors.

We have elected to test our hypothesis using a transgenic approach, which allows us to test the role of LMO4 in the context of the whole animal. We have made significant progress towards this goal. We have decided to create four sets of transgenic mice: one in which LMO4 is overexpressed, one in which LMO4 is converted into a "superactivator", one in which LMO4 activity is inhibited, and a fourth one in which we have overexpressed a dominant negative form of the LMO4-associated protein, CLIM.

a/b. Creation and testing of transgenic plasmids and microinjection of oocytes for establishing transgenic lines.

We successfully created transgenic mice for (1) overexpression of LMO4, (2) overexpression of LMO4/VP-16 activation domain fusion, (3) overexpression of LMO4/engrailed repression domain fusion, and (4) overexpression of a dominant negative CLIM molecule. Analyses of mammary gland expression showed that we established 3 lines expressing MMTV-Engrailed-LMO4, 3 lines expressing MMTV-VP16-LMO4, and 2 lines expressing MMTV-dominant negative-Clim. For the MMTV-LMO4 line, we did not obtain any lines showing significant transgene expression. With the construction of the MCF-7 cells, which overexpress LMO4 in a conditional manner, we were able to address the same question by alternative means as described above.

c. Breeding and analyses of transgenic lines.

The MMTV-LMO4 lines did not exhibit significant transgene expression in the mammary gland, thus preventing further analyses of this mouse strain. In contrast, we have been able to analyze mice from the other three transgenic lines. We have not observed a clear phenotype in the MMTV-VP16-LMO4 and MMTV-dominant negative-Clim lines. In contrast, we have obtained interesting data with the MMTV-Engrailed-LMO4 mice. To test the effect of the Engrailed-LMO4 molecule on mammary gland development, we placed it under control of the MMTV promoter (Fig. 3A in (1)), which directs high expression in epithelial cells of mammary glands in transgenic mice and has been extensively used for this purpose (9-12). The fusion protein was HA tagged to allow its immunodetection in mammary glands. Of five transgenic lines, three independent lines expressed the transgene in mammary gland epithelial cells. Expression of the transgene was found both in virgin and pregnant mammary glands (Fig. 3C in (1)) and was predominantly nuclear (Fig. 3C in (1)).

To evaluate the effects of expressing the Engrailed-LMO4 fusion protein in mammary gland epithelial cells, we examined mammary gland development by whole mount analyses in transgenic mice and compared them to wild-type littermates. Development of transgenic mammary glands of virgin mice was normal at 3 to 4 weeks (data not shown), but at 6 weeks a mild delay in the progression of ductal development was evident (Fig. 3D in (1)). At 8 weeks, most transgenic mammary glands were normal (Fig. 3D in (1), compare WT panel and lower TG panel at 8 weeks), although we did observe occasional abnormality at that stage (Fig. 3D in (1), compare WT panel and upper TG panel at 8 weeks). These data indicate that the Engrailed-LMO4 fusion protein causes a transient delay in mammary gland development of virgin mice. In pregnant transgenic mice, a clear delay in alveolar development was evident at day 5.5 (Fig. 4A and B in (1)); this delay, however, was later overcome and by day 15.5, lobuloalveolar development was essentially normal (Fig. 4B in (1), middle panels). No abnormalities were observed during lactation (Fig. 4B in (1), right panels) and transgenic females were able to nurse normal-size litters. In conclusion, expression of the dominant negative Engrailed-LMO4 fusion protein in the mammary glands of mice results in the slowing of ductal development in virgin mice and a transient inhibition of alveolar development during pregnancy. These results are consistent with our hypothesis derived from the expression analyses and indicate that LMO4 is likely to play roles to promote invasion and/or proliferation of mammary gland epithelial cells.

Objective #3. **Identify and characterize protein partners for LMO-4 in human breast tissue.**

While LMO and LIM homeobox proteins are similar in that they are both localized to the nucleus, there is no evidence to suggest that the biological activity of LMOs is through direct DNA-binding. Insight into the biochemical mechanisms of actions for LMO proteins came from studies of LMO1 and LMO2 in the hematopoietic system where it was found that LMO2 interacts strongly with the bHLH domain of TAL1 (13) and GATA factors (14). These proteins, as well as Clim2, exist in a complex in erythroid cells. These experiments suggest a model in which LMO factors can be tethered to DNA by associating with DNA binding proteins, thus allowing the co-regulator CLIM to interact with transactivators that do not contain a covalently linked LIM domain.

We therefore propose that a LMO4 and Clim2 containing complex regulates gene activity in breast epithelial cells by associating with unidentified DNA-binding protein(s). The goal of the proposed experiments is to identify such factor(s). Specifically, we are interested in determining whether LMO4 may interact with transcription factors or nuclear oncoproteins that have been shown to regulate differentiation and proliferation in normal and neoplastic breast.

a/b. Construction of yeast two hybrid libraries and screening with LMO-4 bait.

In a screen of a human breast library we isolated CLIM-2, human DEAF-1, the DNA-binding factor, Zn43 (1) and the splicosome, protein M4 and SPF27 (2). So far LMO factors have not been implicated in regulation of splicing, but recent data suggest that transcription factors may play a role in regulation of splicing. In addition, it is highly interesting that the gene expressing one of these factors, SPF27, was found to be highly amplified in human breast carcinoma cell lines. This gene has also been referred to as DAM1 (DNA amplified in mammary carcinoma) because it was isolated in screens designed to identify transcripts upregulated in human carcinoma cell lines (3).

c. Characterization of potential positive interacting factors.

We have characterized LMO4-interacting factors by stably transfecting both LMO4 and Clim2 into MCF-7 breast cancer cell lines. The LMO4 protein is tagged with Myc and the Clim2 protein is tagged with HA, thus allowing specific immunoprecipitation of these proteins from breast cancer cell lines. For these studies, we have used Tetracyclin inducible vectors. For further characterization of interacting proteins, a new task, (d), was added.

d. **To use immunoprecipitations of tagged LMO4 and Clim2 proteins to identify potential interacting proteins.** (New task)

This task was added to the original proposal based on a request dated November 13, 2002, which was granted by the Army Breast Program. The LMO2 oncogene, which is highly related to LMO4 is known to interact with GATA factors (14). This suggested the possibility that LMO4 might also interact with GATA factors. Of GATA factors, GATA3 has been shown to be expressed in breast cancer cells (15). We therefore evaluated its expression during mammary gland development in the mouse (Fig. 9) and demonstrated that GATA3 is expressed throughout mammary gland development, and especially highly during pregnancy. In addition, we tested whether LMO4 is capable of interacting with GATA3, using co-immunoprecipitations of extracts from the MCF-7 cells stably expressing Myc tagged LMO4. In this assay, we were able to demonstrate an *in vivo* interaction between GATA3 and LMO4 (Fig. 10). In summary, LMO4 may act by associating with GATA3 in normal and malignant mammary epithelial cells. In future experiments, we plan to characterize this interaction further.

KEY RESEARCH ACCOMPLISHMENTS DURING LAST YEAR

1. Definition of LMO4 and Clim2 gene expression during mammary gland development.
2. Demonstration that LMO4 expression in breast cancer cells is regulated by the breast cancer oncogene ErbB2/Her2/Neu.
3. Demonstration that LMO4 is overexpressed in a subset of human breast cancers.
4. Creation of stable breast cancer cell lines expressing tagged LMO4, DN-Clim and Clim2 proteins, using the Tet-off system.
5. Defining the genes altered by the expression of DN-Clim in breast cancer cells.
6. Defining the transcriptional mechanism by LMO4 in breast cancer cells.
7. Identifying several target genes of LMO4 using microarray analyses, many of which are involved in oncogenesis.
8. Showing that MMTV-Engrailed-LMO4 mice exhibit defective ductular development in virgin mice and defective alveolar development in pregnant mice.
9. Demonstrating expression of GATA3 in mammary glands of mice.
10. Demonstrating an in vivo interaction between LMO4 and GATA3.

REPORTABLE OUTCOMES TO DATE

1. Development of antisera
2. Transgenic mouse models for LMO expression

3. Permanent breast cancer cell lines expressing tagged LMO4, DN-Clim and Clim2 in a conditional manner
4. A fellowship award (BC000553) was funded based on work on this project. This grant from the Army Med Research & Development Command, entitled "Functional Analysis of LIM Domain Proteins and Co-Factors in Breast Cancer", supports Dr. Ning Wang. Total amount is $150,000 over three years.
5. A fellowship award, "Combined Biology and Bioinformatics Approaches to Breast Cancer" (W81XWH-04-1-0483), was funded the Army Med Research & Development Command to my postdoc Zhongxian Lu, based in part on progress related to this project. Total amount $300,000 over three years.
6. Manuscript: Wang, N., Kudryavtseva, E., Chen, I., Sugihara, T.M., McCormick, J., and **Andersen, B.** 2003. Expression of an Engrailed-LMO4 fusion protein in mammary epithelial cells inhibits mammary gland development in mice. *Oncogene,* 2004: 23, 1507-1513.
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8. Abstract: Wang, N., & **Andersen, B.** 2003. The potential role of a new LIM factor, LMO4, in breast cancer. California Breast Cancer Research Program meeting in San Diego, California, September.
9. Abstract: Wang N, Kudryavtseva E, Chen I, McCormick J, Sugihara TM, Ruiz R, **Andersen B.** Heregulin/HER2 regulation of LMO4 in breast cancer cells. *Endocrine Society Annual Meeting,* New Orleans, June 16 – 19, 2004 (Abstract P2-281).

In addition, based on our work, we have two manuscripts in preparation and will acknowledge the suppor to the Army Breast Cancer Research Program.

CONCLUSIONS

In summary, we have made significant progress on all three specific aims. Our results show that LMO-4 expression is associated with undifferentiated breast epithelial cells such as those found during mid-pregnancy and in breast cancer. The major achievements are (1) the finding that interfering with LMO4 in breast epithelial cells leads to inhibition of ductular and alveolar development in mice, (2) the identification of several LMO4 target genes, many of which are involved in oncogenesis, (3) the identification of GATA3 as an LMO4-interacting transcription factor, and (4) the demonstration that LMO4 acts in breast cancer cells to disrupt transcriptional complexes containing the co-factor Clim2. Our findings strengthen the hypothesis that overexpression of LMO4 may contribute to breast carcinogenesis.

With our work, we hope to generate new ideas about treatment of breast cancer, thus impacting on reducing the human/economic cost of breast cancer. I sincerely thank the Army for their support, and I am convinced that our work will play a role in solving the breast cancer problem.

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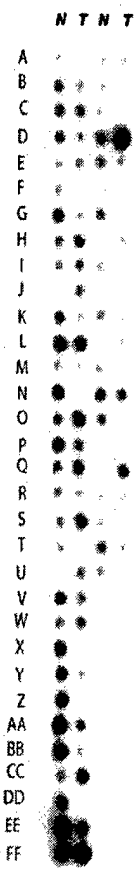

Figure 1. Analysis of LMO4 expression in human breast cancer cases. cDNA synthesized from breast cancer RNA was spotted on a nylon membrane. The membrane was hybridized with ^{32}P-labeled probe specific for LMO4. For each tumor (T) sample there is a matched normal (N) sample from a normal region of the same breast. For example, in row D there are samples from two patients and the patient represented in two rows of the right exhibit striking overexpression of LMO4 in the tumor compared to the normal breast.

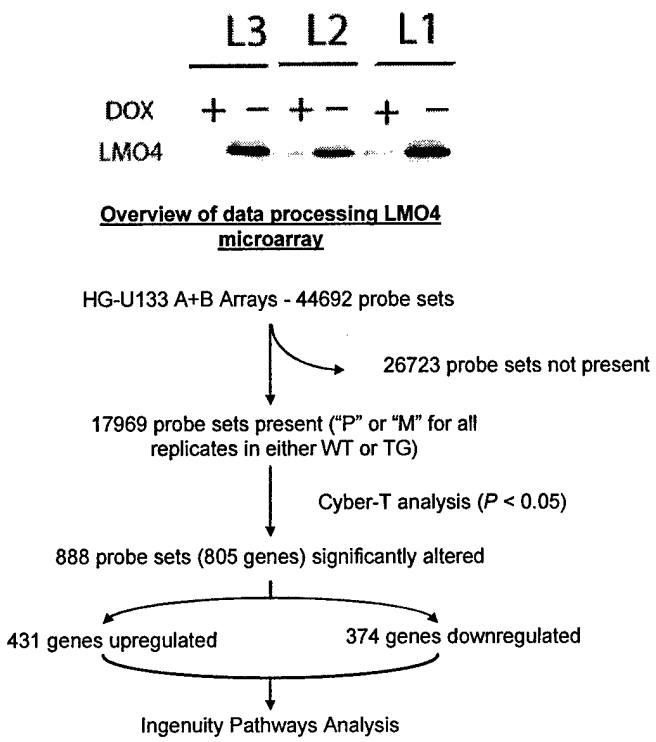

Figure 2. Microarray analysis of gene expression induced by LMO4 in tet-off MCF7 cell lines. Top panel shows that LMO4 is expressed in conditional manner in three different cell lines (L1, L2 and L3), and lower panel shows overview of data processing of the LMO4 microarray results.

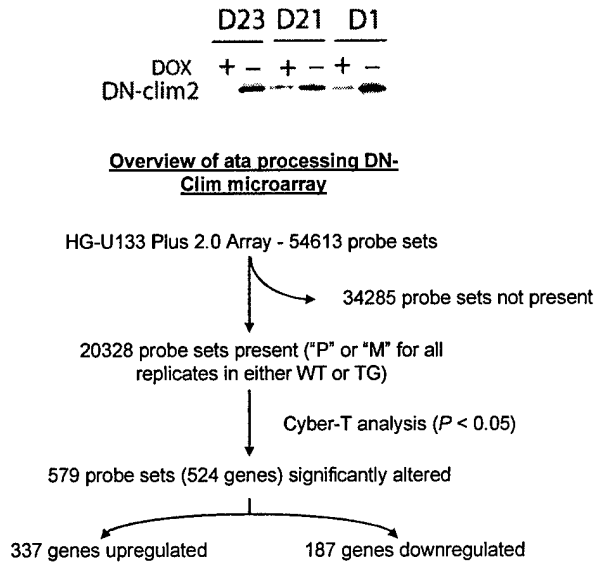

Figure 3. Microarray analysis of gene expression induced by DN-Clim2 in tet-off MCF7 cell lines. Top panel shows that DN-Clim2 is expressed in a conditional manner in three different cell lines (D1, D21 and D23), and lower panel shows overview of data processing of the DN-Clim2 microarray results.

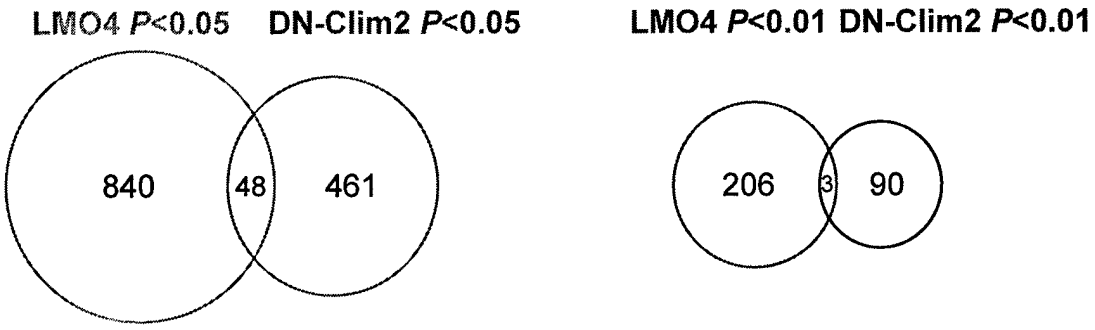

Figure 4. Overlap between genes regulated by LMO4 and DN-Clim2 in our microarray datasets.

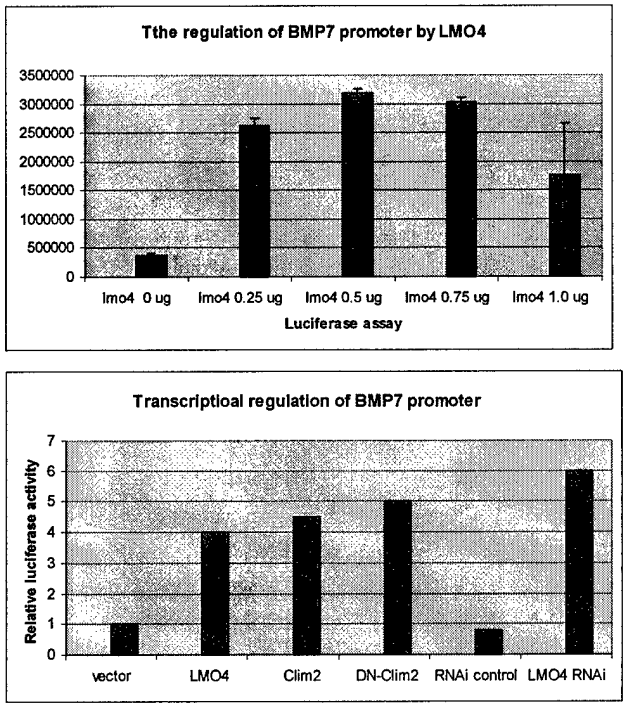

Figure 5. Transcriptional regulation of the BMP7 promoter. Top panel shows that LMO4 can increase BMP7 promoter activity in dose dependent fashion; lower panel shows that LMO4, Clim2, DN-Clim2, and LMO4 RNAi all can increase the BMP7 promoter activity.

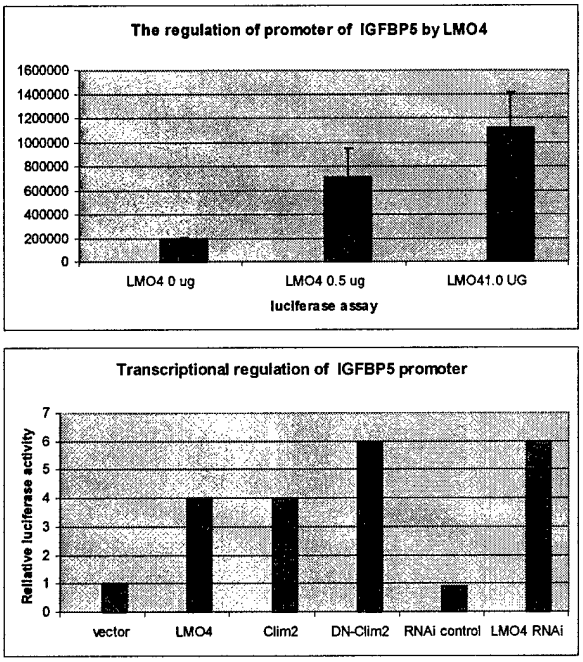

Figure 6. Transcriptional regulation of the IGFBP5 promoter. Top panel shows that LMO4 can increase IGFBP5 promoter activity in dose dependent fashion. Low panel shows that LMO4, Clim2, DN-Clim2, and LMO4 RNAi can increase the IGFBP5 promoter activity.

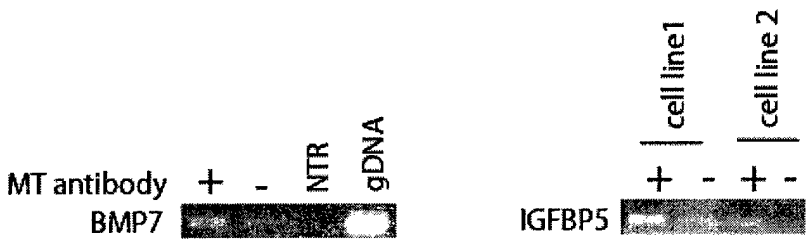

Figure 7. Chromatin imunoprecipitation assays. Cellular chromatin of inducible LMO4 MCF7 cell lines was crosslinked with formaldehyde after LMO4 induction, protein – DNA complexes were incubated with myc antibody and isolated by immunoprecipitation. PCR was performed for the amplification of BMP7 and IGFBP5 promoters. The left panel shows that LMO4 can bind to the BMP7 promoter, and the right panel shows that LMO4 can bind to the IGFBP5 promoter. Negative controls were isolated by immunoprecipitation without antibody (-), NTR: non-template control, g DNA: genomic DNA.

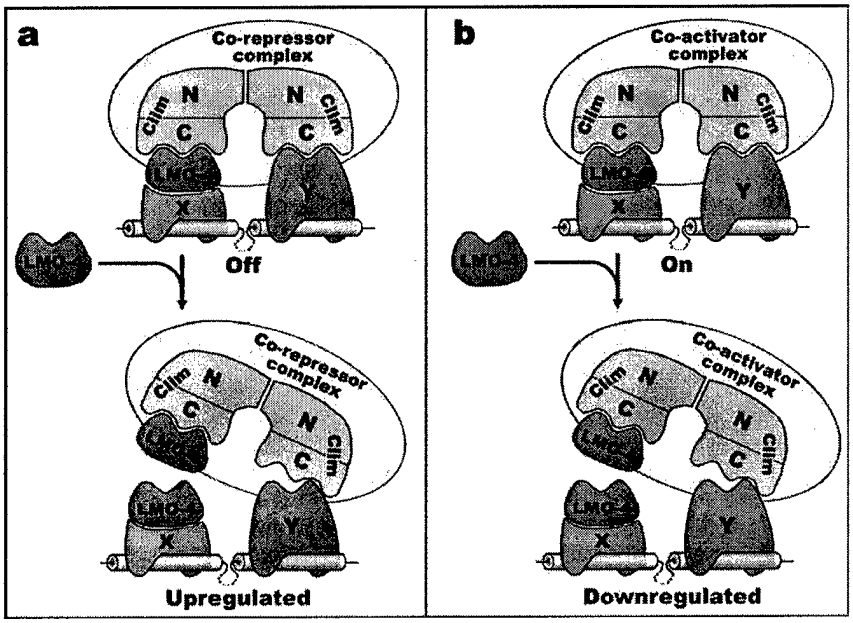

Figure 8. A new model for how LMO4 acts at a transcriptional level in breast cancer cells. LMO4 disrupts Clim-containing complexes. When these complexes contain repressors, the disruption leads to activation of gene expression, and when these complexes contain co-activators, the disruption results in repression of gene expression

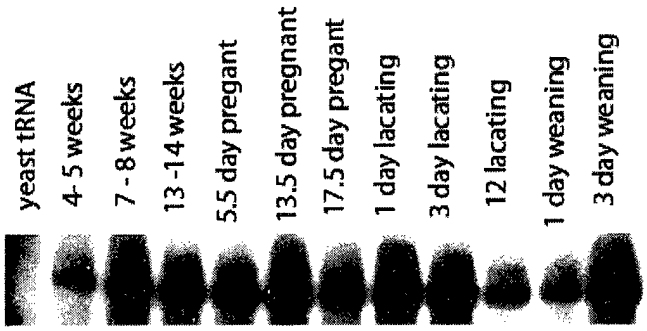

Figure 9. Expression of GATA3 in mammary glands from the indicated stages using RNAse protection assays.

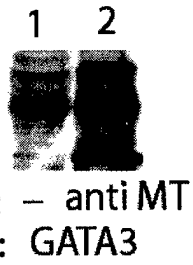

Figure 10. Co-immunoprecipitation showing interaction between LMO4 and GATA3 in MCF-7 breast cancer cells. Extracts from MCF-7 cells expressing Myc tagged LMO4 were analyzed by SDS gel electrophoreses without (lane 1) or after immunoprecipitation with a Myc antibody (lane 2). Western blot was probed with GATA3 antibody. Immunoprecipitation with Myc antibody precipitates GATA3, indicating interaction between LMO4 and GATA3.

Probe set ID	Accession	Gene symbol	Gene name	*P* value	Fold
201369_s_at	NM_006887	ZFP36L2	zinc finger protein 36, C3H type-like 2	2.86E-05	1.82
212593_s_at	N92498	PDCD4	programmed cell death 4	1.14E-04	2.00
215771_x_at	X15786	RET	ret proto-oncogene	1.23E-04	1.91
202428_x_at	NM_020548	DBI	diazepam binding inhibitor	1.62E-04	1.57
204326_x_at	NM_002450	MT1X	metallothionein 1X	2.06E-04	1.73
211259_s_at	BC004248	BMP7	bone morphogenetic protein 7	2.50E-04	2.21
201334_s_at	AB002380	ARHGEF12	Rho guanine nucleotide exchange factor (GEF) 12	2.58E-04	1.76
224671_at	AL571373	MRPL10	mitochondrial ribosomal protein L10	1.01E-03	1.58
202948_at	NM_000877	IL1R1	interleukin 1 receptor, type I	1.11E-03	1.86
204573_at	NM_021151	CROT	carnitine O-octanoyltransferase	1.22E-03	1.97
226722_at	BE874872	FAM20C	family with sequence similarity 20, member C	1.27E-03	1.68
225433_at	AU144104	GTF2A1	general transcription factor IIA, 1, 19/37kDa	1.31E-03	1.50
40093_at	X83425	LU	Lutheran blood group (Auberger b antigen included)	1.55E-03	1.56
204112_s_at	NM_006895	HNMT	histamine N-methyltransferase	1.78E-03	1.66
200028_s_at	NM_020151	STARD7	START domain containing 7	1.92E-03	1.48
217009_at	AL121974	PGK2	phosphoglycerate kinase 2	2.00E-03	2.36
201242_s_at	BC000006	ATP1B1	ATPase, Na+/K+ transporting, beta 1 polypeptide	2.40E-03	1.60
209240_at	AF070560	OGT	O-linked N-acetylglucosamine (GlcNAc) transferase	2.53E-03	1.47
223000_s_at	AF172398	F11R	F11 receptor	2.54E-03	1.35
212525_s_at	AA760862	H2AFX	H2A histone family, member X	2.63E-03	1.63

Probe set ID	Accession	Gene symbol	Gene name	*P* value	Fold
233305_at	AF193756	EFCBP1	EF hand calcium binding protein 1	1.92E-06	-2.38
231713_s_at	NM_018255	STATIP1	signal transducer and activator of transcription 3 interacting protein 1	2.21E-04	-1.47
233208_x_at	AA583986	CPSF2	cleavage and polyadenylation specific factor 2, 100kDa	2.36E-04	-1.68
229558_at	AI927643	MGC16824	esophageal cancer associated protein	3.80E-04	-1.81
233588_x_at	BE561798	HKE2	HLA class II region expressed gene KE2	6.99E-04	-1.91
238346_s_at	AW973003	NCOA6IP	nuclear receptor coactivator 6 interacting protein	8.11E-04	-1.82
225415_at	AA577672	BBAP	rhysin 2	8.40E-04	-1.42
238496_at	AA741074	WHSC1L1	Wolf-Hirschhorn syndrome candidate 1-like 1	1.14E-03	-2.51
212444_at	AA156240	RAI3	retinoic acid induced 3	1.16E-03	-1.51
225496_s_at	N21426	SYTL2	synaptotagmin-like 2	1.33E-03	-1.34
218462_at	NM_025065	RPF1	RNA processing factor 1	1.47E-03	-1.64
207626_s_at	NM_003046	SLC7A2	solute carrier family 7, member 2	1.52E-03	-1.60
220319_s_at	NM_013262	MYLIP	myosin regulatory light chain interacting protein	1.71E-03	-1.51
236300_at	BF698797	PDE3A	phosphodiesterase 3A, cGMP-inhibited	1.78E-03	-1.74
225152_at	BF940944	ZNF622	zinc finger protein 622	2.45E-03	-1.39
208430_s_at	NM_001390	DTNA	dystrobrevin, alpha	2.70E-03	-1.60
223211_at	BC001627	HPCL2	2-hydroxyphytanoyl-CoA lyase	2.86E-03	-1.42
205774_at	NM_000505	F12	coagulation factor XII (Hageman factor)	2.92E-03	-1.52
232337_at	AK000770	B3GNT7	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 7	3.16E-03	-1.54
240027_at	BF062244	LIN7A	lin-7 homolog A (C. elegans)	3.16E-03	-2.14

Table 1. List of the top genes upregulated and downregulated by LMO4 in MCF7 cells. Top panel represents upregulated genes by LMO4 and lower panel represents downregulated genes by LMO4.

Function	Network Genes	*P* value
proliferation	AKT1, CEBPA, CTNNB1, ETS2, **GADD45A**, **GATA2**, NR2C2, RET, TIAM1	8.29E-04
	CD47, CXCR4, DMTF1, IL1R1, ITGB5, MAX	4.90E-05
	BMP7, E2F3, IGFBP5, **ITCH**, **OCLN**, TCF3	2.88E-03
	GRN, SKP2	2.85E-02
	IGFBP2, MTA1	2.85E-02
apoptosis	AKT1, BCL6, **CDC42**, CEBPA, CTNNB1, ETS2, **GADD45A**, HNRPA1, **PAK2**, RET, TCF7L1, TIAM1	9.40E-06
	CD47, CXCR4, MAX, **ODC1**	2.46E-03
	CFLAR, **TNFRSF18**	1.42E-05
	ACVR1B, BMP7, **SMAC**, TCF3	9.01E-03
invasion	AKT1, **CDC42**, CTNNB1, ETS2, TIAM1	2.37E-03
adhesion	CTNNB1, TIAM1	1.49E-02
migration	CD47, CXCR4, ITGB5, MAX	8.33E-08
	CD151, **MDK**	2.70E-03
transformation	DMTF1, MAX, **ODC1**	2.95E-05

Table 2. Ingenuity Pathways Analysis of LMO4 microarray data. The results show that the pathways significantly affected by LMO4 are those involved in cell proliferation and apoptosis.

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Expression of an engrailed-LMO4 fusion protein in mammary epithelial cells inhibits mammary gland development in mice

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LIM domain factors and associated cofactors are important developmental regulators in pattern formation and organogenesis. In addition, overexpression of two LIM-only factors (LMOs) causes acute lymphocytic leukemia. The more recently discovered LMO factor LMO4 is highly expressed in proliferating epithelial cells, and frequently overexpressed in breast carcinoma. Here we show that while LMO4 is expressed throughout mammary gland development, it is dramatically upregulated in mammary epithelial cells during midpregnancy. The LMO coactivator Clim2/Ldb1/NLI showed a similar expression pattern, consistent with the idea that LMO4 and Clim2 act as a complex in mammary epithelial cells. In MCF-7 cells, LMO4 transcripts were upregulated by heregulin, an activator of ErbB receptors that are known to be important in mammary gland development and breast cancer. To test the hypothesis that LMO4 plays roles in mammary gland development, we created an engrailed-LMO4 fusion protein. This fusion protein maintains the ability to interact with Clim2, but acts as a dominant repressor of both basal and activated transcription when recruited to a DNA-regulatory region. When the engrailed-LMO4 fusion protein was expressed under control of the MMTV promoter in transgenic mice, both ductular development in virgin mice and alveolar development in pregnant mice were inhibited. These results suggest that LMO4 plays a role in promoting mammary gland development.
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Introduction

The LIM motif, a cysteine-rich zinc-coordinating domain that mediates protein–protein interactions, was originally discovered as a component of homeodomain transcription factors (reviewed in Bach, 2000). A second class of LIM domain transcription factors, composed almost entirely of two tandem LIM domains, is referred to as LIM-only (LMO) proteins. Two members, LMO1 and LMO2, are oncoproteins found at sites of chromosomal translocations in acute T-cell leukemia (Rabbitts *et al.*, 1999). LMO proteins do not bind DNA directly, but regulate gene transcription by associating with other transcription factors. This model is supported by studies showing that LMOs, through their LIM domains, exist in a stable complex with helix loop helix (HLH) heterodimeric partner proteins that include TAL1(SCL)/E12 (Valge-Archer *et al.*, 1994; Wadman *et al.*, 1994, 1997; Osada *et al.*, 1995, 1997; Larson *et al.*, 1996; Visvader *et al.*, 1997; Ono *et al.*, 1998; Bao *et al.*, 2000; Herblot *et al.*, 2000; Mead *et al.*, 2001), and GATA factors (Osada *et al.*, 1995; Wadman *et al.*, 1997; Ono *et al.*, 1998; Mead *et al.*, 2001). In addition, LIM domains of the LIM homeodomain and LMO proteins interact strongly with cofactors, including the coactivators Clim1 and Clim2/Ldb1/NLI (Agulnick *et al.*, 1996; Jurata *et al.*, 1996; Bach *et al.*, 1997, 1999; Visvader *et al.*, 1997), which confer transcriptional activation and promote synergism between DNA-binding proteins (Bach, 2000).

Based on the prominent expression of Clim2 in proliferating epithelial cells of the epidermis and hair follicles, we discovered LMO4 as a Clim2-interacting protein in the epidermis (Sugihara *et al.*, 1998). LMO4, simultaneously discovered by other laboratories (Grutz *et al.*, 1998; Kenny *et al.*, 1998), is the main LIM domain factor expressed in proliferating epithelial cells of the epidermis and hair follicles (Sugihara *et al.*, 1998). Interestingly, the human *LMO4* gene was initially cloned from a breast cancer cDNA library (Racevskis *et al.*, 1999), and subsequent studies showed it to be overexpressed in more than half of all invasive breast carcinomas (Visvader *et al.*, 2001). Furthermore, LMO4 and Clim2 overexpression interfered with differentiation of cultured mammary epithelial cells (Visvader *et al.*, 2001).

The goals of our studies were to establish a dominant-negative LMO4 molecule that can be used to repress transcription of LMO4 target genes and to study the

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biological function of LMO4 in the mammary gland *in vivo*. We show that fusion of the repression domain from the *Drosophila* engrailed homeobox protein (Han and Manley, 1993) to LMO4 creates a strong transcriptional repressor, capable of interfering with basal and activated transcription. Expression of this fusion molecule under the MMTV promoter in mammary glands of transgenic mice leads to inhibition of ductular and alveolar development, suggesting that LMO4 is involved in progression of mammary gland development.

LMO4 is upregulated in mammary epithelial cells during midpregnancy and by heregulin in MCF-7 breast cancer cells

To gain insights into the role of LMO4 in mammary gland biology, we assessed its expression in mouse mammary gland and breast cancer cell lines. In contrast to a previous study employing Northern blot analyses on total RNA (Visvader *et al.*, 2001), the sensitive RNAse protection assays show that LMO4 transcripts are easily detected in virgin mammary glands and that expression levels remain relatively stable from age 4 weeks to 14 weeks (Figure 1a). However, there is dramatic upregulation of LMO4 in mammary glands from midpregnancy, with levels falling late in pregnancy (Figure 1a), and a moderate increase in LMO4 levels during early lactation (Figure 1a). Clim2 levels are coordinately regulated during mammary gland development, with the highest levels found in midpregnancy (Figure 1a), consistent with the idea that LMO4 and Clim2 act as a complex. *In situ* hybridization studies on mammary gland sections show that LMO4 is primarily expressed in ductular and alveolar epithelial cells (Figure 1b). Consistent with the RNAse protection assay experiments, LMO4 levels are high at day 14.5 and lower at day 18.5 (Figure 1b). The surge in LMO4/Clim2 transcript levels during midpregnancy suggests an especially important function at this developmental stage, characterized by dramatic epithelial cell proliferation and stromal invasion.

In three different human breast cancer cell lines, LMO4 transcript levels vary from high in the estrogen receptor-negative MDA-MB-231, intermediate in the estrogen receptor-negative MDA-MB-453, to low in the estrogen receptor-positive MCF-7 (Figure 1c). Estradiol did not increase LMO4 expression in MCF-7 cells (Figure 1c), consistent with findings in human breast cancer indicating that LMO4 is especially characteristic for estrogen receptor-negative tumors (Gruvberger *et al.*, 2001). In contrast to the coordinately regulated expression of LMO4 and Clim2 during normal mammary gland development (Figure 1a), Clim2 levels remain constant in breast cancer cell lines that express high levels of LMO4 transcripts (Figure 1c) and protein (Figure 1d). These results suggest that relative overexpression of LMO4 compared to Clim2 may be important for LMO4 actions in breast cancer. Since LMO4 may be localized to the cytoplasm under certain conditions (Kenny *et al.*, 1998), we evaluated its cellular distribution in breast cancer cells by generating MCF-7 cells stably expressing an myc-tagged (MT) LMO4. In these cells, LMO4 is restricted to the nucleus (Figure 1e). In contrast to the lack of estrogen regulation, LMO4 expression is stimulated by the ErbB ligand heregulin, which is known to be important for alveolar maturation and proliferation (Figure 1f). Heregulin is thought to act through ErbB2-containing heterodimers (Stern, 2003) and its effect was partially blocked by an ErbB2 antibody (Figure 1g), suggesting a role for ErbB2 in heregulin-mediated upregulation of LMO4.

The observation that LMO4 may be downstream of heregulin/ErbB2 is consistent with findings that the mesenchymally expressed heregulin α, like LMO4, is strikingly upregulated in midpregnancy (Yang *et al.*, 1995). In addition, heregulin and the ErbB2/ErbB3/ErbB4 receptors, which have growth-stimulatory roles (Krane and Leder, 1996; Aguilar *et al.*, 1999), are particularly important for alveolar morphogenesis (Yang *et al.*, 1995; Jones *et al.*, 1996, 1999; Jones and Stern, 1999; Li *et al.*, 2002). ErbB2 is also overexpressed in 15–40% of breast cancer cases, where it is associated with increased invasiveness and metastasis, as well as poor prognosis (Slamon *et al.*, 1989; Eccles, 2001). Our findings suggest the possibility that LMO4 may participate in heregulin/ErbB signaling in the mammary gland.

An engrailed-LMO4 fusion protein is capable of protein–protein interactions and acts as a strong transcriptional repressor

LMO4 forms a complex with Clim coactivators in epithelial cells and is thought to be recruited to DNA-binding proteins, resulting in transcriptional activation of target genes. We hypothesized that fusing the *Drosophila* engrailed transcriptional repression domain to LMO4 would create a dominant-negative molecule capable of suppressing LMO4 target genes (Figure 2a). When fused to heterologous transcription factors, the engrailed repression domain confers strong transcriptional repression. This quality was successfully used to obtain insights into the biological function of a spectrum of transcriptionally active molecules, including c-Myb (Taylor *et al.*, 1996), *Xenopus* tailless (Hollemann *et al.*, 1998), GATA factors (Sykes *et al.*, 1998; Dasen *et al.*, 1999; Liu *et al.*, 2002), homeobox factors iroquois3 (Kudoh and Dawid, 2001) and RaxL (Chen and Cepko, 2002), and β-catenin (Montross *et al.*, 2000).

To test whether the engrailed-LMO4 fusion protein was capable of protein–protein interactions, we performed co-immunoprecipitation assays in HEK293T cells transfected with expression plasmids encoding tagged LMO4 and Clim2, as well as fusion proteins with LMO4 and Clim2. As expected, Clim2 antiserum precipitated LMO4 in cells transfected with Clim2 and LMO4 (Figure 2b). In cells cotransfected with tagged VP16-Clim2 and engrailed-LMO4 fusion proteins, both proteins could be precipitated independent of whether the precipitating antibody was directed against VP16-MT-

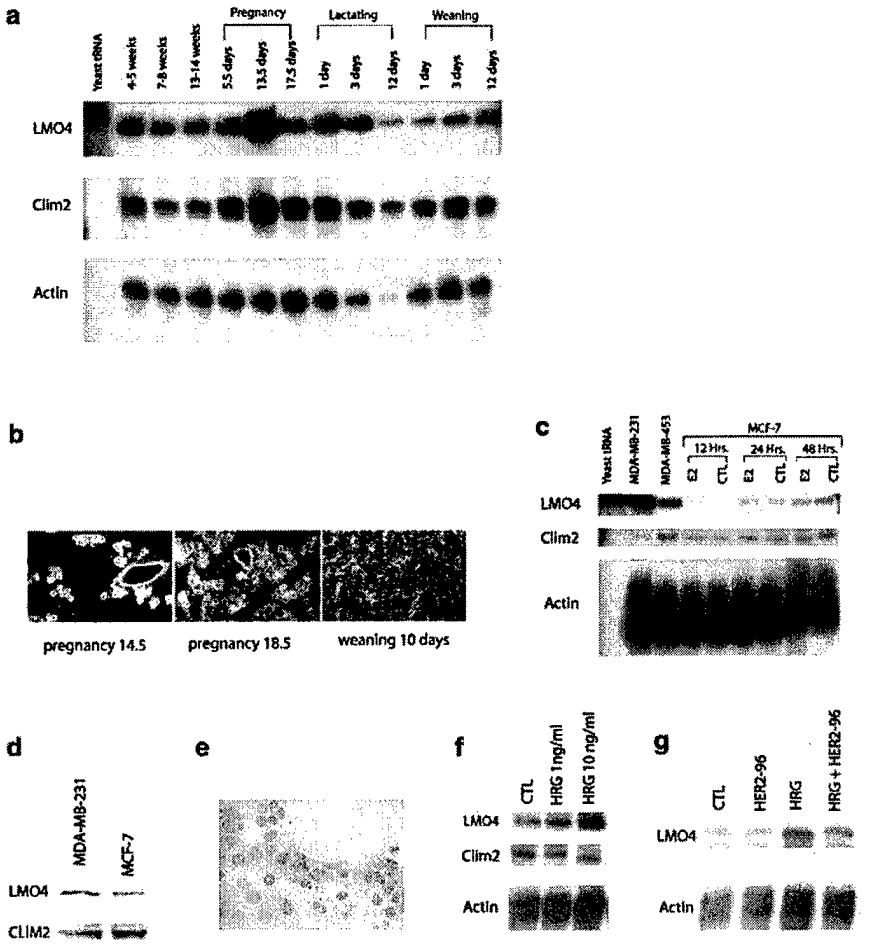


Figure 1 Expression of LMO4 and Clim2 during mammary gland development and in response to regulators of mammary gland development. (**a**) RNAse protection assays showing expression of LMO4 (top panel), Clim2 (middle panel), and β-actin (bottom panel) during the indicated stages of mammary gland development. (**b**) *In situ* hybridization study showing expression of LMO4 in mammary glands at day 14.5 of pregnancy (left panel), day 18.5 of pregnancy (middle panel), and day 10 after weaning (right panel). ^{35}S-labeled cRNA probes specific for mouse LMO4 were applied to formalin-fixed tissue, as described (Sugihara *et al.*, 1998). (**c**) RNAse protection assays showing expression of LMO4 (top panel), Clim2 (middle panel), and β-actin (lower panel) in the indicated breast cancer cell lines and with estradiol (E2) treatment (20 ng/ml) for the indicated times. MCF-7 cells were grown in the presence of phenol red-free media and charcoal-stripped serum. (**d**) Western blot of whole-cell extracts from MDA-MB-231 and MCF-7 cells, using rat LMO4 antibody (Sum *et al.*, 2002) and rabbit Clim antisera (Bach *et al.*, 1999). (**e**) Immunolocalization of LMO4 in MCF-7 cells stably expressing myc-tagged LMO4. After fixing with formalin, slides were incubated with a myc antibody and signal detected with peroxidase. (**f**) RNAse protection assays showing expression of LMO4 (top panel), Clim2 (middle panel), and β-actin (lower panel) in MCF-7 cells after heregulin treatment with the indicated concentrations for 24 h. MCF-7 cells were maintained in serum-free media. Similar effects were observed after 48 h treatment (data not shown). (**g**) RNAse protection assays showing expression of LMO4 (top panel) and β-actin (lower panel) in MCF-7 cells after treatment for 20 h with heregulin and ErbB2-blocking antibody (Clone Her2-96, Sigma). RNA isolation and RNAse protection assays were carried out as previously described (Andersen *et al.*, 1997), using ^{32}P-labeled cRNAs specific for mouse and human LMO4, Clim2, and β-actin

Clim (Figure 2c, left panel) or HA-engrailed-LMO4 (Figure 2c, right panel). We conclude that the fusion of the *Drosophila* engrailed repression domain to LMO4 does not interfere with its ability to interact with Clim proteins.

Since natural target genes for LMO4 are unknown, we tested the effectiveness of the engrailed-LMO4 fusion in a GAL reporter system, where we monitored the transcriptional activity of a luciferase reporter gene under the control of GAL DNA-binding sites and a minimal promoter (Sugihara *et al.*, 1998) (Figure 2d). While LMO4-GAL (Figure 2d, panel 2) has little effect on the basal activity of the promoter, engrailed-LMO4-GAL (Figure 2d, panel 3) represses transcription of the reporter gene 29-fold. Furthermore, engrailed-LMO4-GAL could completely overcome a 105-fold activation conferred by the recruitment of a Clim-VP16 fusion protein (Figure 2d, compare panels 5 and 6). Clim alone is a weak activator in this system and the Clim-VP16 fusion protein is used because the viral VP16 transactivation domain can confer strong transactivation to heterologous proteins. In summary, these experiments suggest that an engrailed-LMO4 fusion protein can repress both basal and activated expression of LMO4 target genes, and that this fusion molecule may be useful to test the biological functions of LMO4.

Expression of the engrailed-LMO4 fusion protein in mammary gland epithelial cells of mice interferes with mammary gland development

To test the effect of the engrailed-LMO4 molecule on mammary gland development, we placed it under control of the MMTV promoter (Figure 3a), which has been extensively used to direct a high expression in epithelial cells of mammary glands in transgenic mice (Muller *et al.*, 1988; Guy *et al.*, 1992; Kitsberg and Leder, 1996; Krane and Leder, 1996). Three independent lines expressed the transgene in mammary gland epithelial cells. Expression of the transgene was found both in virgin and pregnant mammary glands (Figure 3b), and by immunohistochemistry with an HA antibody expression was predominantly nuclear (Figure 3c). The relatively constant level of the transgene expression (Figure 3b) is probably because the transgene in this line is upregulated at the very end of pregnancy, as has been described for other MMTV transgenic mice (Jones *et al.*, 1999). We examined mammary gland development by whole mount analyses in transgenic mice and compared them to wild-type littermates. Development of transgenic mammary glands of virgin mice was normal at 3–4 weeks (data not shown), but at 6 weeks a mild delay in the progression of ductal development was evident (Figure 3d). At 8 weeks, most transgenic mammary glands were normal (Figure 3d, lower TG panel at 8 weeks), although we did observe occasional abnormality at that stage (Figure 3d, upper TG panel at 8 weeks).

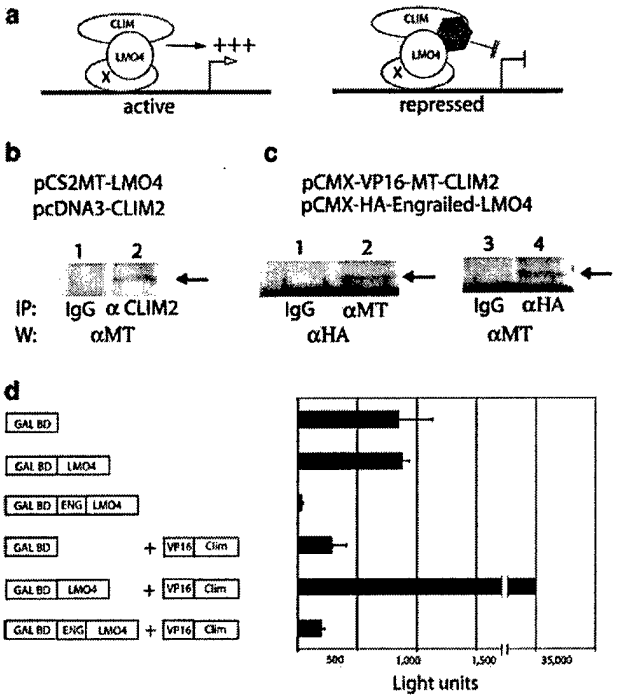
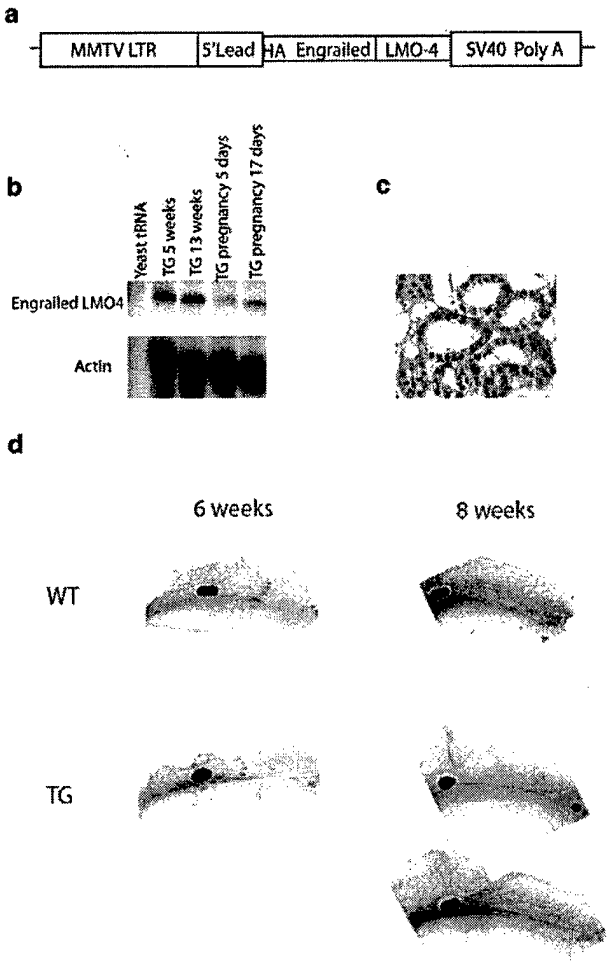


Figure 2 Interactions between Clim2 and LMO4 fusion proteins. (**a**) A model for the activity of the engrailed-LMO4 fusion protein. Under normal condition (left panel), LMO4/Clim complexes are thought to be recruited to promoters of target genes by associating with DNA-binding proteins (indicated as X), resulting in transactivation. Recruitment of the engrailed-LMO4 fusion proteins to the same complexes should result in transcriptional repression. (**b**) Immunoprecipitation of cell lysates from HEK293T cells transfected with expression plasmids encoding myc-tagged (MT) LMO4 and Clim2, using IgG (lane 1) and Clim antibody (Bach *et al.*, 1999) (lane 2). Western blot was probed with MT antibody (Invitrogen). (**c**) Immunoprecipitation of cell lysates from HEK293T cells (Sugihara *et al.*, 2001) transfected with expression plasmids encoding HA-tagged engrailed-LMO4 and myc-tagged VP16-Clim2, using IgG (lanes 1 and 3), MT antibody (lane 2), and HA antibody (lane 4). Western blots were probed with the indicated antibodies. The HA-engrailed-LMO4 fragment was generated in the mammalian expression vector pCMX by fusing the repression domain representing amino acids 2–299 of the Drosophila engrailed gene (Han and Manley, 1993) to a HA tag at the N-terminus and the full-length LMO4 coding sequence at the C-terminus. The pCMXGAL-LMO4 and pCMXGAL-engrailed-LMO4 plasmids contain the full-length LMO4 cDNA and the HA-engrailed-LMO4 fusion protein linked to the GAL DNA-binding domain. The pCMXVP16-Clim plasmid contains the C-terminal LIM-interaction domain of Clim1 (Bach *et al.*, 1999) linked to the VP16 transactivation domain. (**d**) The indicated GAL DNA-binding domain fusion proteins and VP16 fusion proteins were transfected into HEK293T cells with a GAL-luciferase reporter plasmid, using calcium-mediated gene transfer (Sugihara *et al.*, 1998). The results, expressed as light units, represent the mean and standard deviation from triplicate transfections. IP, immunoprecipitation; W, Western blot



These data indicate that the engrailed-LMO4 fusion protein causes a transient delay in mammary gland development of virgin mice.

In pregnant transgenic mice, a clear delay in alveolar development was evident at day 5.5 (Figure 4a and b); this delay, however, was later overcome, and by day 15.5 lobuloalveolar development was essentially normal (Figure 4b). No abnormalities were observed during lactation (Figure 4b) and transgenic females were able to nurse normal size litters. In conclusion, expression of the dominant-negative engrailed-LMO4 fusion protein in the mammary glands of mice results in the slowing of ductal development in virgin mice and a transient inhibition of alveolar development during pregnancy, suggesting that LMO4 plays roles in both ductular and alveolar development *in vivo*.

The phenotype of the MMTV-HA-engrailed-LMO4 mice may be distinct from the expected phenotype of LMO4 null mice. First, the engrailed-LMO4 fusion protein can suppress the expression of LMO4 target genes both under basal and activated conditions. In contrast, deletion of the LMO4 gene is likely to affect only the genes where LMO4 is actually participating in regulated transcription. Second, while it is generally thought that LMOs in combination with Clims are involved in transactivation, LMO4 may also participate in repression of certain genes, as has been suggested with BRCA1-mediated transcriptional activity (Sum *et al.*, 2002). The engrailed-LMO4 fusion protein would not be expected to affect these genes since they are already repressed. Finally, LMO4 may also act by binding to and sequestering other proteins in solution, a process the engrailed-LMO4 fusion protein would not be expected to inhibit. Such mechanisms have been proposed for the effect of *Drosophila* lmo in the fly wing (Zeng *et al.*, 1998).

The effect of engrailed-LMO4 expression in mammary glands was most clearly observed in early pregnancy, but the defect was overcome towards the end of pregnancy. Such defects, in which mammary gland development is slowed but not blocked, have been previously described in other genetically modified mice such as those with mutations in the ErbB2 gene (Stern, 2003). However, it is not possible to conclude that the role of LMO4 is restricted to ductular development in virgin mice and alveolar development in early pregnancy, because it is impossible to determine which levels of transgene expression are required to block endogenous LMO4 protein levels. Despite these limitations of the dominant-negative approach, our results strongly

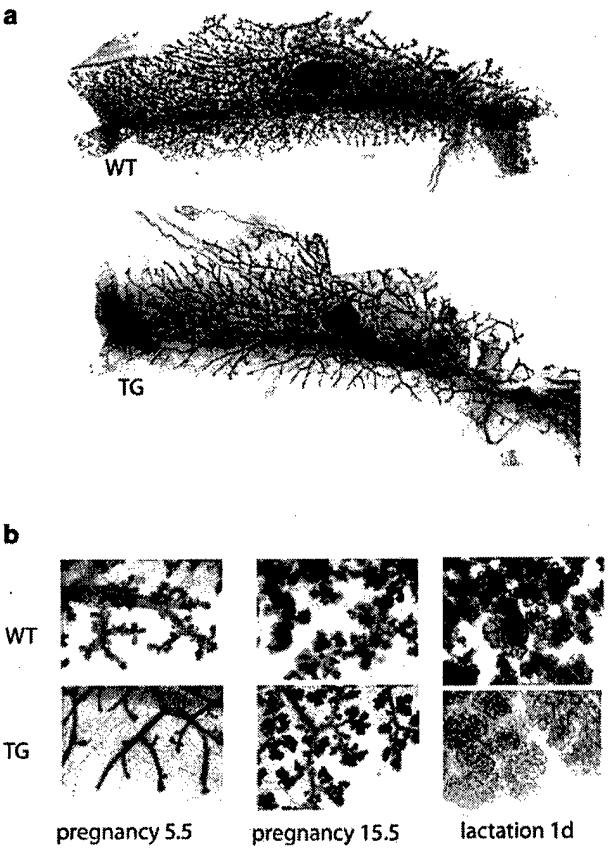


Figure 4 Effect of the engrailed-LMO4 fusion protein on mammary gland development during pregnancy. (**a**) Whole mount overview of mammary glands from 5.5-day pregnant mice comparing wild-type (WT) and transgenic (TG) mice. (**b**) Whole mount analyses in higher magnification from the indicated developmental stages. All magnifications are the same. Representative results from the analyses of 10 (5.5 day), six (15.5), and four (lactating) transgenic mice are shown

Figure 3 Effect of the engrailed-LMO4 fusion protein on mammary gland development in virgin mice. (**a**) A schematic of the transgene. The MMTV-HA-engrailed-LMO4 plasmid was created by cloning the HA-engrailed-LMO4 fragment into the *Eco*R1 site of the MMTV-SV40-BSSK plasmid (Leder *et al.*, 1986). To generate transgenic mice, the plasmid was cut with *Xho*1 and *Spe*1 to remove extraneous sequences, and the purified DNA fragment was then injected into fertilized CB6F1 oocytes, which were implanted into pseudopregnant mice. Of 13 mice born, five contained the MMTV-HA-engrailed-LMO4 sequences, as assessed by PCR with oligonucleotides specific for MMTV sequences. Of these five lines of founder mice, three (lines #1, 2, and 7) expressed the transgene, as assessed by immunohistochemistry with HA antibody on pregnant mammary glands. The three expressing lines were expanded by breeding into CB6F1 mice. Experiments were carried out with transgenic mice derived from lines #1, 2, and 7, which showed a comparable level of abnormality in mammary gland development. (**b**) RNAse protection assays showing expression of the engrailed-LMO4 transgene from line #7 at the indicated developmental time points. The probe, which corresponded to the *Drosophila* engrailed part of the fusion molecule, was specific because no signal was observed in mammary glands from wild-type mice (data not shown). (**c**) HA immunostaining of mammary gland (day 1 of lactation) from MMTV-HA-engrailed-LMO4 mice. Immunostaining of wild-type littermates gave no staining with the HA antibody (data not shown), indicating that the staining is specific. The mammary glands were fixed for 1 h at room temperature in a solution composed of six parts of ethanol, three parts of water, and one part of formaldehyde, followed by storage in 70% ethanol at 4°C. Paraffin-embedded tissue sections were stained with a monoclonal HA antibody (Covance) using peroxidase. (**d**) Whole mount staining of the fourth inguinal mammary glands from MMTV-HA-engrailed-LMO4 (TG) mice and littermate wild-type (WT) controls at the indicated developmental stages. Representative results from analyses of 16 (6 weeks) and three (8 weeks) TG mice are shown. The mammary glands were dissected, processed as a whole mount, fixed and stained with hematoxylin as described (Brisken *et al.*, 1999), and photographed at the same magnification

support roles for LMO4 in both ductular and alveolar development. Moreover, the dominant-negative LMO4 is a promising tool to evaluate the possible role of LMO4 in signaling pathways and in breast cancer.

The etiology of sporadic breast cancers is multifactorial and thought to involve stepwise mutations in several oncogenes and tumor-suppressor genes. The findings described in this paper are of importance because there are parallels between mammary epithelial cells during pregnancy and in breast cancer, and the LMO4 gene is frequently overexpressed in breast cancer. While neoplastic breast epithelial cells clearly have properties distinct from epithelial cells of the developing breast, the two also share similarities such as active proliferation and lack of terminal differentiation (Rudland *et al.*, 1998). Our studies – showing high expression of LMO4 during a stage in mammary gland development when there is active proliferation and stromal invasion, and the inhibition of these processes with a dominant-negative LMO4 molecule – lend support for the idea that LMO4 upregulation may contribute to the tumorigenic characteristics of mammary epithelial cells (Visvader *et al.*, 2001).

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THE POTENTIAL ROLE OF A NEW LIM FACTOR, LMO4, IN BREAST CANCER

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Many properties of breast cancer cells, including increased proliferation and invasion, are common to epithelial cells of the developing mammary gland, suggesting that understanding of developmental control in normal mammary glands may provide important insights into the biology of breast cancer. This notion is supported by work in many organ systems, demonstrating that subversion of developmental control genes plays roles in carcinogenesis. LIM domain factors and associated co-regulators are important developmental regulators involved in pattern formation and organogenesis in a wide spectrum of organisms, including mammals. We isolated a LIM only factor, LMO-4, which is highly expressed in epithelial cells, including mammary epithelium. Interestingly, LMO factors are known to be oncogenic in lymphocytes where their overexpression causes acute lymphocytic leukemia.

We have studied expression of LMO-4 in mammary glands of mice and found that it is most highly expressed in proliferating mammary epithelial cells during pregnancy, suggesting that the LMO-4 gene may play a role in proliferation. Since LMOs do not bind to DNA it is likely that they regulate transcription by interacting with DNA-binding proteins and transcriptional co-regulators. To search for such factors, we have screened a human breast cDNA library with LMO-4 as bait in the yeast two hybrid system and found several potential interacting partners, including DNA-binding proteins, Clim/Nli/Ldb co-regulators and a splicing factor previously shown to be amplified in breast cancer cell lines. To test the role of LMO-4 in mammary gland biology, we have generated three lines of transgenic mice expressing under control of the MMTV promoter a) wild-type LMO-4, b) LMO-4 fused to the VP-16 transactivation domain and c) LMO-4 fused to the engrailed repression domain. Whole mount mammary gland analyses of these transgenic mice is in progress and preliminary results will be presented. Analyses of the EST databases indicate that LMO-4 is highly expressed in mammary carcinomas and we are in the process of evaluating its expression in breast cancer.

We conclude that LMO-4 may be an important regulator of mammary epithelial cells and propose a hypothesis that its high level expression in mammary tumors may play a role in mammary carcinogenesis.

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Heregulin/Her2 Regulation of LMO4 in Breast Cancer Cell.

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The growth factor receptor Her2 is overexpressed in about 40% of breast cancer cases, where it is thought to induce tumorigenicity and metastasis of breast cancer cells. The ligand heregulin (HRG) activates the Her2 receptor and its downstream signal transduction via induction of heterodimeric complexes of Her2 with Her3 or Her4. LIM domain factors and associated co-factors are important developmental regulators in pattern formation and organogenesis. The LMO (LIM-only) family consists of four members (designated LMO1-LMO4), each of which contains two tandem LIM domains. Overexpression of LMO1 and LMO2 leads to acute lymphocyte leukemia. The more recently discovered LMO factor LMO4 is highly expressed in proliferating epithelial cells and frequently overexpressed in breast cancer, suggesting that LMO4 may contribute to pathogenesis of breast cancer. The regulation of LMO4 is poorly understood. In this study, we explored the regulation of LMO4 mRNA expression in MCF-7 cells by HRG. We treated MCF-7 cells with HRG β1(20ng/ml) and measured the LMO4 transcript levels at different time points by RNase protection assays. LMO4 transcripts were up-regulated by HRG and this effect could be partially blocked by an Her2 antibody, suggesting a role for Her2 in HRG-mediated up-regulation of LMO4. The observation that LMO4 may be downstream of HRG/Her2 is consistent with the findings that mesenchymally-expressed HRG, like LMO4, is strikingly upregulated in mid-pregnancy. Furthermore, overexpression of a dominant negative LMO4 (engrailed LMO4) under the MMTV promoter inhibits ductular development in virgin mice and alveolar development in pregnant mice. In summary, our findings suggest the possibility that LMO4 may participate in HRG/ErbB signaling in the mouse mammary gland and breast cancer.

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